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Challenges in using allylthiourea and chlorate as specific nitrification inhibitors

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Abstract

Allylthiourea (ATU) and chlorate (ClO_3^-) are often used to selectively inhibit nitritation and nitratation. In this work we identified challenges with use of these compounds in inhibitory assays with filter material from a biological rapid sand filter for groundwater treatment. Inhibition was investigated in continuous-flow lab-scale columns, packed with filter material from a full-scale filter and supplied with NH_4^+ or NO_2^-. ATU concentrations of 0.1-0.5 mM interfered with the indophenol blue method for NH_4^+ quantification leading to underestimation of the measured NH_4^+ concentration. Interference was stronger at higher ATU levels and resulted in no NH_4^+ detection at 0.5 mM ATU. ClO_3^- at typical concentrations for inhibition assays (1-10 mM) inhibited nitratation by less than 6%, while nitritation was instead inhibited by 91% when NH_4^+ was supplied. On the other hand, nitratation was inhibited by 67-71% at 10-20 mM ClO_3^- when NO_2^- was supplied, suggesting significant nitratation inhibition at higher NO_2^- concentrations. No chlorite (ClO_2^-) was detected in the effluent, and thus we could not confirm that nitritation inhibition was caused by ClO_3^- reduction to ClO_2^-. In conclusion, ATU and ClO_3^- should be used with caution in inhibition assays, because analytical interference

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and poor selectivity for the targeted process may affect the experimental outcome and compromise result interpretation.

Keywords:
drinking water, ammonium, nitrite, ATU, chlorate, inhibition

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1. Introduction

Aerobic nitrification is a two-step process consisting of the oxidation of ammonium to nitrite (nitritation) and of nitrite to nitrate (nitratation). Ammonium oxidizing bacteria (AOB) and ammonium oxidizing archaea (AOA) are responsible for nitritation (Martens-Habbena et al., 2009; Martens-Habbena and Stahl, 2011; Prosser, 1989; Prosser and Nicol, 2008), whereas nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate (Lees and Simpson, 1957). The two nitrification steps are linked and take place simultaneously as nitratation uses the product of nitritation, but can be uncoupled and investigated individually using compounds that inhibit one of the two steps. Inhibition is the result of blockage or inactivation of the normal catalytic cycle of the enzyme responsible for a specific function, i.e. nitritation or nitratation (McCarty, 1999).

Allylthiourea (ATU) is commonly used to inhibit nitritation, by targeting the ammonia monooxygenase action and chelating the copper in the active site, ultimately hindering its function (Bedard and Knowles, 1989). Nitritation inhibition has been used in micropollutant biodegradability studies (Batt et al., 2006; Falas et al., 2012; Shi et al., 2004; Zhou and Oleszkiewicz, 2010; Rattier et al., 2014) and in studies investigating nitritation kinetics (Munz et al., 2010) and activity (Dapena-Mora et al., 2007).

Chlorate (ClO_3^-) has been used to inhibit nitrite oxidation and inhibition is

presumably a result of chlorate reduction to chlorite (ClO_2^-) (Hynes and Knowles, 1983). This reduction is catalyzed by nitrate reductase, which is actually the same enzyme that is responsible for nitrite oxidation, operating in the reverse direction (Hynes and Knowles, 1983). As a result, chlorate inhibition is assumed to be specific for nitritation. Chlorate inhibition has also been widely used when quantifying the ammonium oxidation potential of biomass (Belser and Mays, 1980; ISO, 2012).

Specific inhibition by ATU and chlorate has been used for decades in a variety of environmental systems, ranging from soils to activated sludge, marine sediments and pure cultures. Although similar behavior with other oligotrophic systems was expected, we experienced challenges with the use of these compounds in inhibition assays with filter material from biological rapid sand filters for groundwater treatment. The aim of this work was therefore to investigate, adress and report these challenges to avoid the potential occurrence of experimental artifacts in future work.

2. Materials & Methods

2.1. Investigated rapid sand filter and filter material sampling

A rapid sand filter at Islevbro waterworks (Copenhagen, Denmark operated by Hofor A/S) was used for the experimental investigations. The filter had been operating for 30 years prior to the experiments without filter material replacement. Filter influent contained on average 0.13 mg/L NH_4^+-N, which was completely nitrified to NO_3^-, 9.25 mg/L O_2, 1.93 mg/L NVOC, and 0.1 mg/L Fe^{+2} (Tatari et al., 2013). Filter material was collected from the top 5 cm using a sterilized 1 L stainless steel container attached to an extendable aluminum rod. Three random

horizontal locations were sampled and the collected filter material was mixed to form a composite sample. After sampling, the sand was stored wet at 4 °C for less than 7 days before the inhibition assays.

2.2. Inhibition assays

The inhibitory effect of ATU and ClO_3^- was investigated by monitoring the nitrification activity of the biomass on the collected filter material in a lab-scale assay. Due to the long operating time of the sampled full- scale filter, the filter material had an already established active nitrifying community, with AOB densities of 31 cells/m^3 filter material and a nitrification capacity above 223 g NH_4^+-N/m^3 filter material/d (Tatari et al., 2016). The experimental system employed small plexiglas columns (5 cm bed height, 2.6 cm inner diameter), packed with the collected filter material and operated continuously (Tatari et al., 2013). Effluent water from the waterworks was supplemented with 1-2 mg/L NH_4^+-N as NH_4Cl (Sigma-Aldrich, 254134) or 1 mg/L NO_2^--N as $NaNO_2$ (Sigma-Aldrich, S2252) and the inhibitory compounds at concentrations described later, and was fed at the inlet of the columns. Alkalinity in the influent water was high (5.4 meq/L as HCO_3^-), so no additional alkalinity was added to the substrate water. The influent flowrate was constant at 39 mL/h giving a hydraulic retention time (HRT) of 2.3 h in the system, as determined experimentally by salt (NaCl) tracer tests. Column effluent was recirculated at a ratio of 50 to 1 to impose complete mixing in the bulk phase in the system (Tatari et al., 2013).

The columns were packed and started-up with only the N-substrate in the influent, running therefore as controls for a day. On the second day of operation, the column effluents were sampled twice (with 3-4 h in between) and were analyzed for the NH_4^+, NO_2^- and NO_3^- concentrations. After sampling of the con-

trols, ATU (N-Allylthiourea, Merck chemicals, 808158) or ClO_3^- ($KClO_3 \geq 99\%$, Sigma-Aldrich, 12634) were added in the influents and continously supplied with the N-substrates. ATU was only added in columns supplied with NH_4^+. ClO_3^- was added in columns supplied with NO_2^- to verify nitratation inhibition, and in columns supplied with NH_4^+ to assess the selectivity of the nitratation inhibition. Concentrations of the two compounds and combinations with the N-substrates are reported in Table 1. The effluents were sampled twice (with 3-4 h in between), 18 h after the addition of the inhibiting compound (at least 8 HRT after onset of application) and were analyzed for NH_4^+, NO_2^- and NO_3^- concentrations. Columns were then emptied and cleaned by high water flow (390 mL/h) for 3-4 h before re-packing with new filter material for the next experiment, unless specified otherwise in Table 1.

Nitritation inhibition (%) was calculated as the difference in NH_4^+ removal during control and test column performance. NH_4^+ removal (%) was calculated by subtracting the effluent from the influent NH_4^+ concentration and normalizing for the influent NH_4^+ concentration. Similarly, nitratation inhibition (%) was calculated as the difference of NO_2^- removal during control and test column performance. NO_2^- removal (%) was calculated as the difference of effluent and produced NO_2^- concentration, after correction for the NO_2^- traces present in the influent water (0.016 mg/L NO_2^--N) and normalization for the produced NO_2^- concentration. The NO_2^- produced by nitritation was calculated as the difference of influent and effluent NH_4^+ concentration.

2.3. Analytical methods

NH_4^+ was quantified colorimetrically with the indophenol blue method (Merk, Spectroquant test kit, 1.14752) and absorbance was measured at 690 nm with a

Table 1: Inhibitory effect of ATU and ClO_3^- at different concentration levels on nitritation and nitratation

Inhibitor	Inhibitor Concentration (mM)	Substrate	Influent Substrate (mg N/L)	% Nitritation Inhibition	% Nitratation Inhibition
ATU	0.10	NH_4^+	1	87	0.0
	0.50[i]		1	87	0.0
	0.50		2	96	0.0
ClO_3^-	0.01	NH_4^+	1	11	1.1
	0.05		1	15	0.0
	0.10		1	28	0.0
	1.00		1	83	1.9
	5.00		1	80	3.1
	10.0		1	85	5.9
ClO_3^-	0.01	NO_2^-	1	-	5.4
	10.0[ii]		1	-	67
	20.0[iii]		1	-	71

[i] Same filter material as in the above experiment (0.1 mM ATU & 1 mg/L NH_4^+). ATU concentration was increased to 0.5 mM at day 3.

[ii] Same filter material as in the above experiment (0.01 mM ClO_3^- & 1 mg/L NO_2^-). ClO_3^- concentration was increased to 10 mM at day 3.

[iii] Same filter material as in the above experiment (10 mM ClO_3^- & 1 mg/L NO_2^-). ClO_3^- concentration was increased to 20 mM at day 4.

spectrophotometer (Merck, DR 2800). Detection limit of the method was 0.05 mg NH_4^+-N. Measured NH_4^+ concentrations in the influents containing ATU were lower than the nominal spiked levels, so NH_4^+ was re-quantified by flow injection adapting the method from Hall and Aller (1992). In brief, the carrier stream was 10 mM NaOH (Sigma-Aldrich, 30620) with 0.2 M Na-citrate (Sigma-Aldrich, 25116) and the receiving stream was 0.1 mM HCl (Sigma-Aldrich, 30721). Both streams were fed by a multi-channel pump (LabConco, Model 140250) at a rate of 6.2 mL/min. 100 μL of sample were manually injected in the carrier stream through an injection valve. NH_4^+ in the sample was converted to gaseous NH_3 due to the basic pH of the carrier stream. The carrier stream entered the gas exchange membrane (Teflon tape), through which NH_3 diffused to the receiving stream and was converted back to the ionic form (NH_4^+) due to the acidic pH. Conductivity of the receiving stream was measured by a conductivity meter (VWR Scientific, Model 1054) and was linearly correlated to the NH_4^+ content of the sample. Conductivity peaks were retrieved and processed with the Clarity software (Data Apex, Advanced Chromatography Data Station). Detection limit of the method was 0.07 mg NH_4^+-N.

NO_3^-, ClO_3^- and ClO_2^- were quantified by Ion Chromatography (Dionex, ICS 1500) using a guard column (Dionex, AG 22) and an analytical column (Dionex, ION PAC AS22). The eluent solution contained 4.5 mM Na_2CO_3 (Sigma-Aldrich, 31432) and 1.4 mM $NaHCO_3$ (Sigma-Aldrich, 71630), and detection was done by suppressed conductivity (Dionex Thermo Scientific, ASRS 300). Retention times were approximately 5.7 min for NO_3^-, 5 min for ClO_3^- and 3.5 min for ClO_2^-. Detection limits were 0.2 mg NO_3^--N/L, 0.01 mM ClO_3^- and 0.01 mM ClO_2^-.

3. Results & Discussion

3.1. ATU interference in NH_4^+ quantification by the indophenol blue method

NH_4^+ quantification by the indophenol blue method resulted in lower NH_4^+ values compared to the concentrations measured by flow injection in all samples where ATU was present (Figure 1). NH_4^+ concentration measured by flow injection in the influent samples matched the nominal spiked concentrations and fitted the N-balance within the standard error of the analytical methods (data not shown).

To exclude a potential matrix effect, 7 standard solutions were prepared by spiking the effluent water from Islevbro waterworks with NH_4^+-N at 0.05-1 mg/L. The standard solutions were analyzed colorimetrically and NH_4^+ was retrieved in all standards within the standard deviation of the method (0.023 mg NH_4^+-N/L). A second series of 4 standard solutions in the same water matrix contained 1 mg/L NH_4^+-N and 0.05-0.5 mM ATU, and was also quantified colorimetrically. Measured NH_4^+-N concentrations were between 0 and 0.80 mg/L, confirming the underestimation of NH_4^+ concentration by the indophenol blue method in the presence of ATU.

Interference by ATU in the flow injection method was excluded by analyzing a series of 6 standard solutions (0.07-1.12 mg/L NH_4^+-N) without or with 0.5 mM ATU, where all measured concentrations matched the nominal standard concentrations.

NH_4^+ analysis by the indophenol blue method did not detect any NH_4^+ in the influents spiked with 0.5 mM ATU, although their nominal NH_4^+ concentration was 1-2 mg/L NH_4^+-N (Figure 1). Interference of ATU with the colorimetric method was stronger at higher ATU concentrations. However, the NH_4^+ concentration from colorimetric determination was not correlated with the actual NH_4^+ concentration

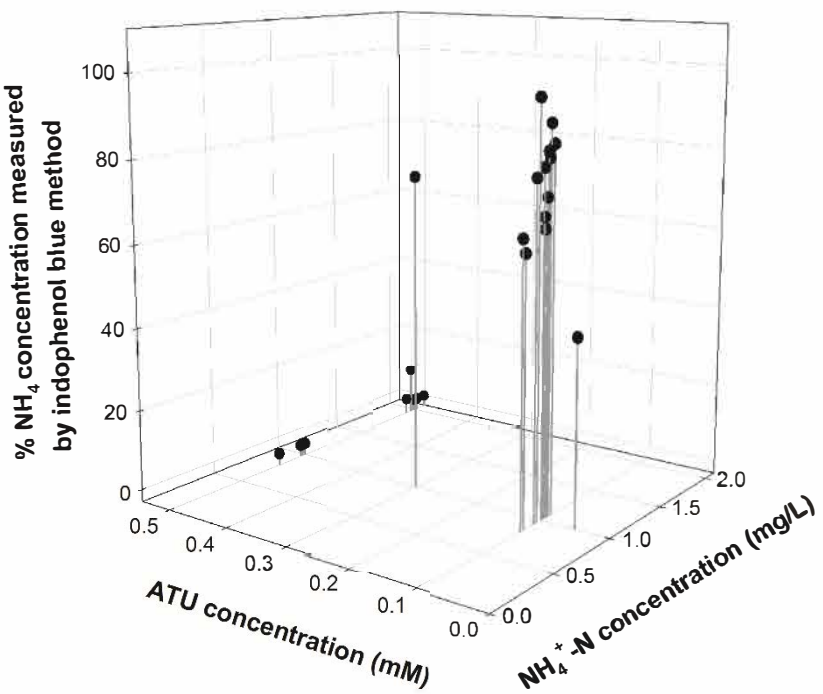

Figure caption

Figure 1: NH_4^+ concentration underestimation by the indophenol blue method when ATU was present in the samples

for a given ATU level, so no correction factor could be calculated.

Although the indophenol blue method has been used in several works to quantify NH_4^+ in ATU inhibited samples (Law et al., 1992; Lehtovirta-Morley et al., 2013; Minzoni et al., 1988), this is the first study to identify and report the analytical interference of ATU with this method. These previous studies however, used ATU concentrations between 0.009 and 0.08 mM, which are lower than the range used in our study. Only Montuelle et al. (2003) using 1.7 mM ATU suggested a potential interference in the NH_4^+ quantification, although they did not not investigate nor specifically attribute the interference to the presence of ATU. ATU concentrations up to 0.08 mM are typically used to inhibit AOB activity, while higher concentrations up to 0.86 mM are relevant for archaeal (AOA) activity inhibition (Santoro and Casciotti, 2011; Hatzenpichler et al., 2008; Taylor et al., 2010). Therefore, when ATU is used to inhibit AOA activity at concentrations higher than 0.08 M the use of the indophenol blue method for NH_4^+ quantification should be discouraged. Erroneous conclusions can arise if only effluent NH_4^+ concentrations are measured as they would be underestimated due to interference, resulting in overestimation of the ATU inhibitory effect.

3.2. Selectivity of ClO_3^- inhibition for nitratation

The efficiency and selectivity of ClO_3^- in inhibiting nitratation was evaluated by experiments with NH_4^+ or NO_2^- as the supplied substrate. During the initial operation as controls, NH_4^+ removal in the columns supplied with NH_4^+ was higher than 98%, and nitratation was complete since the effluent NO_2^--N concentration was below 0.016 mg/L. NO_2^- removal during the control operation of the columns supplied with NO_2^- ranged between 88 and 97%.

Inhibition observed due to ClO_3^- addition is illustrated in Table 1 for the 9

197 combinations of N-substrates and ClO_3^- concentrations. ClO_3^- at 1-10 mM inhib-
198 ited nitritation (80-85%), but not nitratation (< 6%) in the columns supplied with
199 NH_4^+ (Table 1). On the other hand, nitratation was inhibited (67-71%) at 10-20
200 mM ClO_3^- when NO_2^- was the sole influent substrate (Table 1). This differential
201 ClO_3^- effect on nitratation can be possibly related to the different NO_2^- concentra-
202 tion in the columns. At 10 mM ClO_3^-, the effluent NO_2^--N concentration was 0.82
203 mg/L in the column supplied with NO_2^- and 0.02 mg/L in the column supplied
204 with NH_4^+. These effluent concentrations were equal to the NO_2^- concentrations in
205 the columns because of complete mixing in the system. Hence, this indicates that
206 nitratation inhibition was higher at high vs. low NO_2^- concentrations. Our results
207 are in contrast with those of Belser and Mays (1980), who observed a stronger
208 inhibitory effect at low NO_2^- concentrations suggesting a competitive inhibition
209 mechanism. Our results do not support such a mechanism, but indicate that dif-
210 ferent NOB communities may respond differently to ClO_3^-.

211 Nitritation inhibition by ClO_3^- has been observed previously and has been at-
212 tributed to reduction of ClO_3^- to ClO_2^- by NOB (Hynes and Knowles, 1983). We
213 did not detect ClO_2^- in the column effluents (LOD 0.02 mM), while the ClO_3^- added
214 to the influents was retrieved in the effluents (data not shown). We therefore could
215 not confirm the hypothesized reduction of ClO_3^- to ClO_2^- or the reduction to Cl^- as
216 suggested by Xu et al. (2011). This, however, does not exclude the possibility that
217 ClO_3^- was temporarily reduced and oxidized in a cyclic pattern, producing inter-
218 mediates that inhibited nitritation. According to Hynes and Knowles (1983), AOB
219 are presumably much more sensitive than NOB to ClO_2^-, meaning that even low
220 concentrations may inhibit nitritation. These observations ultimately suggest that
221 selectivity of ClO_3^- inhibition towards nitratation may be compromised by specific

experimental factors, such as the NO_2^- concentration and the type of active NOB. Therefore, the ClO_3^- inhibition method to estimate nitrification potential should be tested before use to exclude an eventual significant nitritation inhibition during the assay that may lead to experimental artifacts .

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Addendum

Since the original submission of this article, we have discovered – using 16S rRNA targeted amplicon sequencing and qPCR - that the microbial community on the sand material from the examined rapid sand filters is highly enriched in members of the *Nitrospira* over the *Nitrosomonas* genus (ca 100/1, (Gülay et al., 2016; Tatari et al., 2016)) We subsequently discovered – via metagenomics – that the *Nitrospira* population was dominated by types with the genetic capacity for complete ammonium oxidation (comammox) (Palomo et al., 2016). Our recent work – using stable isotope probing – reveals that comammox *Nitrospira* drive ammonium oxidation in the RSF samples under the examined conditions (Gülay et al., unpublished). Hence, our observations on the behavior of ClO_3^- as a strong inhibitor of nitritation can be explained: even though ClO_3^- is only converted to the toxic ClO_2^- in cells that have an active nitriteoxidoreductase enzyme (NXR, i.e. perform nitratation), the majority of these cells are likely comammox *Nitrospira*, and the toxic ClO_2^- would then not only inhibit most cells in their nitrite oxidation capacity (nitratation) *but also* in their ammonium oxidation capacity (nitritation). The observed 'lack of nitratation inhibition' under ammonium-fed conditions can also be explained: as nitritation inhibition is not 100% complete, there are some cells that are not affected by ClO_3^- ; those cells' nitration capacity would then neither be affected, limited-to-no NO_2^- accumulation would be observed, and absence of nitritation inhibition would be inferred. Yet, we note that ClO_3^- causes strong nitratation inhibition when NO_2^- is provided as sole substrate: this is consistent with the normal mode of action of ClO_3^-, either by inhibiting comammox *Nitrospira* or other NOBs that oxidize extracellular NO_2^-. Clearly, our further observations on the abundance of comammox *Nitrospira* strengthen and refine the conclusion: the selectivity of ClO_3^- as inhibitor of nitratation may be compromised by the type of NOB and is abolished when comammox *Nitrospira* dominate ammonium oxidation in the system. These observations, furthermore, suggests that the response to ClO_3- may serve as an indicator of the contribution of comammox to nitrification.

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